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Rapid Generation of Stable Cell Lines Expressing High Levels of Erythropoietin, Factor VIII, and an Antihuman CD20 Antibody Using Lentiviral Vectors

Lajos Baranyi,[1] Christopher B. Doering,[2] Gabriella Denning,[3] Richard E. Gautney,[3] Kyle T. Harris,[3] H. Trent Spencer,[2] Andre Roy,[1] Hatem Zayed,[1] and Boro Dropulic[1]

Abstract

Lentiviral vectors (LVs) are widely recognized as the most efficient method for the stable delivery of nucleic acid sequences into mammalian cells. Using erythropoietin (EPO), recombinant factor VIII (fVIII), and an anti-CD20 antibody as model proteins, we demonstrate advantages of LV-based gene delivery to achieve high production levels by transduced cells. Highly productive cell clones were able to incorporate up to 100 vector copies per cellular genome, without selection or gene amplification, and were isolated without extensive screening of a large number of clones. The LV transgenes were shown to be distributed throughout the genome, as visualized by fluorescent *in situ* hybridization. High-expressing clones producing 100–200 pg/cell/day of EPO were isolated and characterized. EPO production was demonstrated for at least 5½ months of continuous culture without selection, during which all the clones displayed high levels of glycosylation despite production levels at 10–20 g/liter. To demonstrate the utility of LV technology for multiple classes of proteins, cell lines producing fVIII and an anti-CD20 antibody were also developed. Cell clones demonstrating high levels of fVIII (100 clot units/ml and anti-CD20 antibody as high as 40–100 pg/cell/day) were isolated and characterized. LV-transduced cells and plasmid-transfected cells were compared for protein production per transgene copy. LV-transduced cells produced significantly higher levels of protein per copy of transgene than plasmid-transfected cells did. This study demonstrates the utility of LV technology for rapid generation of highly productive and stable cell lines over conventional plasmid transfection methods, significantly decreasing the time, cost, and risk of the manufacture of proteins and other complex biological molecules.

Introduction

Manufacturing processes limitations have constrained the broader use of recombinant biologics, particularly the lack of consistency associated with the generation of high, stable, and post-transcriptionally uniform protein-producing cell lines using conventional transfection methods (Cumming, 1991; Hooker and James, 1998; Butler, 2006).

There are several issues affecting the development of cell lines for the manufacture of clinically relevant proteins. One is the time necessary for their development, which can take several months and requires the screening of potentially thousands of cell clones to identify one that is highly productive (Wurm, 2004). Another is the stability of the cell clone, which is dependent upon the site of integration of the transgene. Misfolding, aggregation, and variable glycosylation are known factors that affect protein function and safety (Dunker *et al*., 2008; Jenkins *et al*., 2008; Wandinger *et al*., 2008). Glycosylation is considered to be one of the most important factors where insufficient or improper glycosylation critically impacts the functionality of many proteins (Schellekens, 2008; Kresse, 2009) and vaccines (Heinrich, 1990; Larrick and Thomas, 2001; Freire *et al*., 2006; Simerska *et al*., 2009) *in vivo*. Indeed, the development of biosimilars is hampered by the lack of uniformity of recombinant protein production from cell lines (Cumming, 1991; Jenkins, 1995; Hooker and James, 1998; Butler, 2006). Therefore, there is high interest in the development of methods that could potentially improve the consistency and uniformity of the production of biologics from cell substrates.

[1]Lentigen Corporation, Gaithersburg, MD 20878.
[2]Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322.
[3]Expression Therapeutics, LLC, Tucker, GA 30084.

Lentiviral vectors (LVs) have advantages over other gene delivery systems in that they can efficiently transduce dividing and nondividing cells (Naldini, 1996). They can be transduced in a manner that facilitates multiple copy integration per cell. They integrate into areas of open chromatin, the site of active transcription (Schroder *et al*., 2002; Grund *et al*., 2010; Dropulic, 2011). Here, we demonstrate that these features render the LV system highly suitable for generating cell substrates for industrial protein manufacturing.

Three clinically relevant proteins were selected for this study: erythropoietin (EPO), a short human protein; a large, hard-to-produce chimeric human–porcine factor VIII; and a monoclonal antibody, specific for CD20 antigen, widely used for breast cancer treatment and already manufactured in massive quantities.

Recombinant human EPO (Maiese *et al*., 2008), one of the first successful biologics commercialized (Bebbington *et al*., 1992; Lusher *et al*., 1993; Doering *et al*., 2002; Kanda *et al*., 2006; Jurado Garcia *et al*., 2007; Beyer *et al*., 2009), is a 192-amino acid-long protein that is glycosylated with N-acetyl glucosamine at three native N-glycosylation sites. EPO is a well-studied, medically and historically important protein known for its sensitivity to improper glycosylation that affects its biological activity by changing its affinity to its receptor and it's *in vivo* half-life (Jurado Garcia *et al*., 2007; Llop *et al*., 2008). Justified by its importance, there is increasing interest in the manufacturing of EPO biosimilars (Joung *et al*., 2007; Ronco, 2008; Schellekens, 2008; Macdougall and Ashenden, 2009). Here we show that LVs can transduce Chinese Hamster Ovary (CHO) cells with high vector copy numbers per cell, resulting in high levels of properly glycosylated EPO production that was shown to be stable for at least 5½ months of continuous culture without evidence of gene silencing in any of the clones analyzed.

Recombinant human–porcine factor VIII (rhpFVIII) prescribed for the treatment of Hemophilia A has a size of almost 300 kDa and is heavily glycosylated with 20 N-linked and at least 7 O-linked glycans. Low productivity of rhpFVIII from cell substrates has made its production problematic, driving up cost, and consequently keeps prophylactic treatment as an option limited mainly to industrialized countries (Lusher *et al*., 1993). To improve productivity, a series of *rhpFVIII* constructs have been shown to increase fVIII protein production in heterologous, plasmid vector-based expression systems (Doering *et al*., 2002). In this article, we show superior production of these *rhpFVIII* using LV-mediated transduction over transfected plasmid vector-based expression methods, generating cell lines that produce fVIII at significantly higher levels.

Immunoglobulins, like anti-CD20 (Rituximab), dominate the biologics production landscape, and with the rapid improvement of upstream processes, volumetric productivities are higher than ever (Kelley, 2009). The limits, however, have yet to be reached, with specific productivity of hybridomas usually only in the 1–5 pg/cell/day range. More recent recombinant IgG production technologies that rely on genetically modified mammalian cells have improved productivity to approximately 5–20 pg/cell/day, still inferior to plasma cells (20–80 pg/cell/day) (Bebbington *et al*., 1992; Kanda *et al*., 2006; Beyer *et al*., 2009). Here we show that two LVs, each expressing the heavy or light anti-CD20 immunoglobulin chains, were able to efficiently transduce and generate cell clones that produced antibody with specific productivities of 40–100 pg/cell/day. The high frequency of highly productive clones attainable with two LVs (H and L chains on separate vectors) obviates the need to express antibodies and other bicistronic genes from a single vector (Fang *et al*., 2007) and permits the selection of cell clones with heavy-to-light-chain ratios that may not be attainable from single-vector systems.

Materials and Methods

Advanced Dulbecco's modified Eagle/F12 medium (DMEM/F12), AIM-V medium, Opti-MEM medium, heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin solution, GlutaMax, and Dulbecco's phosphate-buffered saline (PBS) were purchased from Invitrogen Life Technologies. Cell transfections were performed using Polyethyleneimine "Max" MW 40,000 (PEI) purchased from Polysciences, Inc. Human fVIII-deficient plasma and normal pooled human plasma Factor Assay Control (FACT) were purchased from George King Biomedical. Automated activated partial thromboplastin reagent was purchased from BioMérieux, Inc. Clotting times were measured using an Start Coagulation Instrument (Diagnostica Stago). Genomic DNA and total RNA from cultured cells were isolated using Qiagen DNeasy Blood & Tissue Kit and RNeasy Mini Kit, respectively. *In vitro* transcribed fVIII RNA standards were generated using the mMessage mMachine T3 Kit by Ambion. Analysis of fVIII transgene integration events and fVIII RNA quantitation was performed using SYBR GREEN PCR Master Mix, TaqMan Reverse Transcription Reagents (RNA quantitation only), and ABI Prism 7000 Sequence Detection System (Applied Biosystems). Oligonucleotides were synthesized by Integrated DNA Technologies. Chemicals were purchased from Fisher Scientific unless otherwise specified.

Southern blot analysis for *rhpFVIII* mRNA expression was performed using the DIG Nonradioactive Nucleic Acid Labeling and Detection System by Roche.

Lentivector production

HIV-based LVs expressing the human EPO transgene were manufactured at Lentigen. Briefly, the *EPO* gene was amplified using the TC12534 as a template vector (Origene). Primers used were GATCATGCGGC GCGCCACCATGGGGGTGC ACGA-ATGTCCT (forward primer) and CAGCTATGACCG CGGCCGCAACTAGAGTCGAGCCT (reverse primer). Polymerase chain reaction (PCR) products were cut with *Asc*I/*Not*I and gel-purified. The purified product was directionally cloned into a Vesicular Stomatitis Virus Glycoprotein pseudotyped Self-Inactivating lentiviral expression vector (Fig. 1A) (Lentigen). Transgene expression was driven by the human elongation factor 1α (EF-1 α) promoter or simian cytomegalovirus (SCMV) promoter, Central Polypurine Tract/Central Termination Sequence, and contained the woodchuck hepatitis post-transcriptional regulatory element. Research-grade LV particles were manufactured by transient transfection using helper constructs. Harvests were concentrated and titers were obtained by performing quantitative PCR using vector specific primers on extracted genomic DNA from transduced 293 cells. Lots with titers $>1\times10^7$ transducing units/ml were released for experiments.

Construction of ReNeo rhpFVIII expression vector

The ET-801 transgene was subcloned into fVIII the ReNeo mammalian expression vector, which has been described previously (Doering *et al*., 2002).

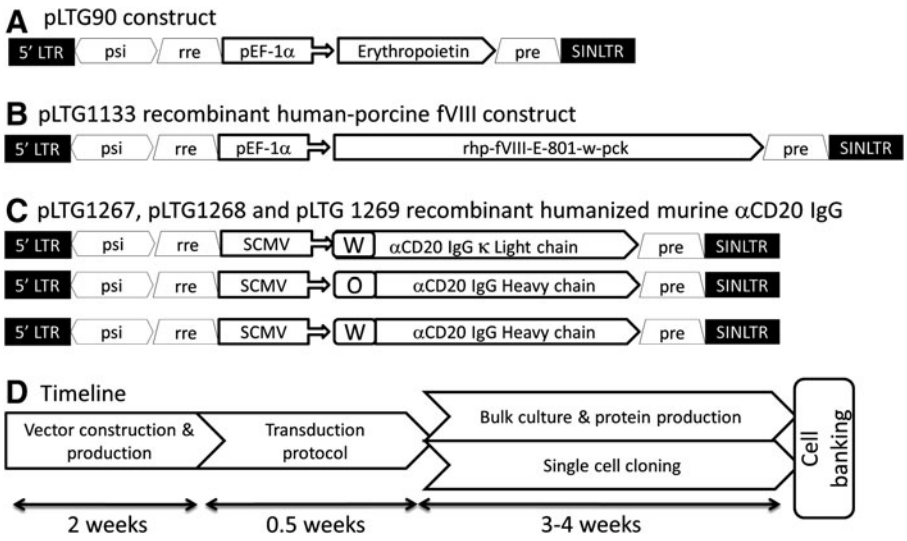

FIG. 1. Construction of lentiviral vectors and cell line development. **(A)** The erythropoietin (UniProtKB/Swiss-Prot entry P01588) encoding gene was amplified using the TC12534 as a template vector (Origene): forward primer, GATCATGCGGC GCGCCACCATGGGGGTGCACGAATGTCCT; reverse primer, CAGCTATGACCG CGGCCGCAACTAGAGTCGAGCCT. Polymerase chain reaction products were cut with *Asc*I/*Not*I and gel-purified. The purified product was directionally cloned into a Self-Inactivating lentiviral expression vector (Lentigen) in frame with elongation factor-1 alpha promoter. **(B)** The recombinant human–porcine FVIII (*rhpFVIII*) gene was cloned into lentiviral backbone along with human elongation factor 1 promoter, without additional antibiotic selection marker to use for cell line development. **(C)** Three vectors were developed for the recombinant anti CD20 humanized murine immunoglobulin production: one vector encoding the light chain and the Simian Cito-megalo Virus promoter; and two constructs for the gamma heavy chain containing either a wild-type (W) murine leader peptide (MRAPA-QIFGFLLLLFPGTRCDI) or an (O) encoded an optimized leader peptide (MRAPAQIFGFLLLLFPGTCFA). **(D)** Timeline for vector construction, production, cell transduction, and cloning. Vector cloning and production is performed over a 2-week period. The cells are then transduced multiple times for 3–4 days, after which the cells are either grown in bulk or undergone limiting dilution and single-cell cloning. Single-cell clones were isolated and banked using standard procedures.

Cell lines and tissue cultures

293FT cells were used for vector production (Invitrogen). *DG44 cells* were obtained from Invitrogen and maintained in ProCho5 serum-free medium. Transductions were performed repeatedly in ProCho medium using 10–150 multiplicity of infection (MOI)/cell repeatedly. The transduced cells were single cells cloned using limiting dilution method. The selected clones were cultured in perfusion cultures using CL1000 disposable bioreactor (Sartorius Stedim North America, Inc.). ProCho5 medium was supplemented with 2 m*M* HT, 2 m*M* GlutaMAX (Invitrogen), and 10 m*M* HEPES (Sigma-Aldrich); pH was set to 7.3. The culture temperature was 37°C, CO_2 5%, and humidity 90%. The inner chamber of the perfusion culture contained 15–20 ml of cell suspension. As an alternative high-density culture method, hollow fiber cultures using FiberCell System's Duet with 50 ml cartridges were also maintained, following the manufacturer's instructions. For seeding and after cell density/viability changes, cells from the harvested media were counted and viability and cell sizes were determined by running 0.5 ml suspension samples on VI-Cell XP cell viability analyzer (Beckman Coulter).

Cell culture, transfection, transduction, and clonal selection

Stable transfection of ET801 vector–Baby hamster kidney-derived (BHK-M; a kind gift from Dr. R. MacGillivray, University of British Columbia, Canada) cells (Funk *et al*., 1990) were grown to 90% confluency in six-well plates containing 2 ml Advanced DMEM/F12 supplemented with 10% FBS, 1% penicillin/streptomycin solution, and 1% GlutaMax (Complete Advanced DMEM/F12). Cells were transfected with 1.5 μg plasmid DNA using PEI. Briefly, plasmid DNA diluted in 200 μl of Opti-MEM was mixed with 6 μg of PEI (1 mg/ml stock) and incubated at room temperature for 20 min. Cells were rinsed twice with 1 ml of Advanced DMEM/F12 supplemented with 10% FBS and 1% GlutaMax (no antibiotics), and the plasmid DNA:PEI mixture was added to the rinsed cells containing 800 μl of Advanced DMEM/F12 supplemented with 10% FBS and 1% GlutaMax (no antibiotics). After overnight incubation at 37°C and 5% CO_2, transfection medium was replaced with 2 ml of Complete DMEM/F12 medium. Transfected cells were cultured for an additional 24 hr before re-plating 100,000 cells onto a 10 cm cell culture plates in 10 ml of Complete DMEM/F12 supplemented with 300 μg/ml Geneticin for clonal selection. Selection medium was replaced every 3–4 days, and visible colonies were picked with a pipette tip and transferred to a 96-well plate approximately 14 days postselection. Clonal cell populations then were expanded for further analysis. Lentiviral transduction was performed as follows: Approximately, 100,000 BHK-M cells were plated onto collagen-1-coated 24-well plates (Biocoat; Becton Dickinson) in Complete Advanced DMEM/F12 and cultured overnight at 37°C with 5% CO_2. Transductions at MOI 30 were performed by incubating lentivirus with cells in a final volume of 500 μl of Complete Advanced DMEM/F12 supplemented with

8 μg/ml of polybrene (Specialty Media). Twenty-four hours post-transduction, virus-containing medium was replaced with 1 ml fresh Complete Advanced DMEM/F12 and transduced cells were allowed to recover by culturing for an additional 1–2 days at 37°C with 5% CO_2. Serial transductions were performed by re-plating previously transduced cells for another round of transduction as outlined above, and transductions were repeated until fVIII activity did not increase significantly from previous transduction (a total of 5 transductions). Clonal isolation was performed by single-cell cloning by limiting dilution into a 96-well plate and visual examination of wells for single cell using cells after five rounds of transduction. After 10 days in culture, single-cell clones were expanded in sequence from the 96-well plate to 48-well, 24-well, 12-well, 6-well, and 10 cm culture plates for further analysis. Each clone was analyzed for fVIII activity, integration events, and transcript expression as described below.

Measurement of fVIII activity

For all *in vitro* studies, fVIII activity was measured using the activated partial thromboplastin reagent-based one-stage coagulation assay in an ST art Coagulation Instrument (Diagnostica Stago) using human fVIII-deficient plasma as the substrate as previously described. Briefly, 5×10^5 cells were plated in duplicate onto a six-well culture plates and cultured overnight at 37°C/5% CO_2 in 2 ml of Complete DMEM/F12. Conditioned medium was then replaced with 2 ml of AIM-V serum-free medium and cultured at 37°C with 5% CO_2 for an additional 24 hr before assaying fVIII activity. All coagulation assays were measured in duplicate from two independent wells, and activity (units/ml) was determined by linear regression analysis of clotting times against a FACT standard curve. Cells were counted at the time of assay in order to express fVIII levels as units/10^6 cells/24 hr.

Measurement of fVIII transgene copy number

Genomic DNA from transduced cells was isolated using the DNeasy Blood & Tissue Kit following the manufacture's protocol for adherent cells in culture. Genomic DNA was quantitated spectrophotometrically at 260 nm using NanoDrop 2000 spectrophotometer (A260 reading of 1 = 50 μg/ml DNA based on an extinction coefficient of DNA in dH_2O). Transgene copy number was determined by real-time quantitative PCR of genomic DNA isolated from transduced cells using A1 porcine-specific fVIII-specific primers: forward primer, 5′-CAG GAG CTC TTC CGT TGG-3′; reverse primer, 5′-CTG GGC CTG GCA ACG C-3′. Transgene copy numbers were determined in a background of 1 μg genomic DNA, and absolute quantitation was determined against a standard curve generated using genomic DNA from a cell line harboring 3 copies/cell of fVIII transgene (as described below). Briefly, PCRs for unknown samples were carried out in 25 μl of total volume containing 1×SYBR Green PCR master mix, 250 μ*M* forward and reverse primers, 50 ng of unknown sample genomic DNA, and 950 ng of HEK-293T genomic DNA. PCRs for standard curve were also in 25 μl of total volume containing 1×SYBR Green PCR master mix, 250 μ*M* forward and reverse primers, and standard genomic DNA ranging from 500,000 to 64 copies generated by serial dilution in a total of 1 μg HEK-293T genomic DNA. Standard curve genomic DNA used for absolute quantitation was derived from a HeLa clonal cell line harboring 3 copies/cell of hybrid human–porcine fVIII transgene, which was generated by lentiviral transduction of HeLa cells followed by cloning by limiting dilution. Real-time PCR was performed by one cycle at 95°C for 10 min followed by 40 amplification cycles of 90°C for 15 sec and then 60°C for 1 min. Postreaction dissociation analysis was performed to confirm single-product amplification by performing a single cycle of 95°C for 15 sec, 60°C for 30 sec, and 95°C for 15 sec. Number of copies per cell was determined using an average value of 8,333 copies per 50 ng of genomic DNA.

Measurement of rhpFVIII transcript expression

Total RNA was extracted from cell lines using RNeasy Mini Kit following manufacturer's instructions using QIA shredder homogenizers. RNA concentrations were determined by absorbance at 260 nm using NanoDrop 2000 spectrophotometer (A260 reading of 1 = 44 μg/ml RNA based on an extinction coefficient for RNA at neutral pH). Porcine fVIII RNA standard, used for absolute quantitation of fVIII transcripts by real-time reverse transcription PCR, was generated as described previously. PCRs were carried out in 25 μl of total volume containing 1×SYBR Green PCR master mix, 300 μ*M* forward and reverse primers, 12.5 units of MultiScribe, 10 units of RNase Inhibitor, and 5 ng of sample RNA. PCRs using porcine fVIII RNA standard also included 5 ng of yeast tRNA to mimic RNA environment of sample RNA using serial dilutions (102–106 transcripts/reaction) as template RNA. The oligonucleotide primers used were located in the fVIII sequence encoding the A2 domain located at positions 2047–2067 for forward primer (5′-ATG CACAGCATCAATGGCTAT-3′) and 2194–2213 for reverse primer (5′-GTGAGTGTGTCTTCATAGAC-3′) of the human fVIII cDNA sequence. Primers recognize both human and porcine fVIII, as sequences in this region are identical between both templates. One-step real-time reverse transcription PCR was performed by incubation at 48°C for 30 min for reverse transcription followed by one cycle at 95°C for 10 min, and 40 amplification cycles of 90°C for 15 sec and then 60°C for 1 min. Postreaction dissociation analysis was performed to confirm single-product amplification by performing a single cycle of 95°C for 15 sec, 60°C for 30 sec, and 95°C for 15 sec. Number of transcripts per cell was determined using an average value of 142.7 cell equivalents per 5 ng of RNA.

Southern blot analysis. Integration events in six plasmid and six lentivirally derived clones were confirmed by Southern blot analysis. Briefly, 10 μg of total genomic DNA was digested overnight with either *Eco*RV or *Avr*II for plasmid and lentiviral clones, respectively. Digested genomic DNA samples were separated on a 0.8% agarose/TAE gel and transferred by capillary action using Saline with Sodium Citrate buffer to a positively charged nylon membrane. After UV-crosslinking, membrane was hybridized overnight with a probe complimentary to the porcine A1 domain. Probe generation, hybridization, and detection were performed per manufacturer's instructions outlined in the DIG Nonradioactive Nucleic Acid Labeling and Detection System (Roche).

EPO enzyme-linked immunosorbent assay. For EPO determination, Quantikine IVD Epo kit (R&D Systems) was

used as described in the insert, except that the culture supernatants were diluted 1,000 to 10 million fold to be in the linear range of the assay.

P24 enzyme-linked immunosorbent assay and PCR. HIV-1 p24 Antigen Capture Assay–Enzyme Immunoassay was performed for the detection of HIV-1 p24 in tissue culture media (Advanced BioScience Laboratories) as described in the insert. Detection of LV copy number was performed using real-time PCR as previously reported.

Western blot. 500 ng of total protein loaded onto a 4–12% Bis-Tris gradient gel (Invitrogen) was run with 1×MOPS buffer (Invitrogen). Samples were run under denatured and reduced conditions at 200 V for 45 min. Samples were transferred to a polyvinylidene difluoride membrane (Invitrogen) and blocked with Super Block Buffer (Pierce) containing 0.05% Tween-20 (Sigma) for 1 hr at room temp. Rabbit antihuman EPO (AB-286-NA; R&D Systems) was diluted 1:1,000 in blocking buffer for 1 hr at room temperature. Blot was washed three times with PBS containing 0.05% Tween-20. Donkey antirabbit IgG HRP secondary antibody (#NA934; Amersham) was used at a 1:10,000 dilution in blocking buffer. Detection was done with Super Signal Kit (Pierce).

Fluorescent *in situ* hybridization. EPO-producing and nontransduced Dg44 cell lines were maintained and expanded in ProCho5 medium. A single passage was performed on each line in order to seed the cells for 12 hr Colcemid treatment for arresting in mitotic division. The cells were harvested, treated with hypotonic solution, and fixed in methanol/acetic acid. In each cell line, we observed a high number of metaphase spreads suitable for fluorescent *in situ* hybridization (FISH) analysis. Metaphase spreads were prepared for the DG44 and DG44 EPO cell lines. Spreads were aged and viewed by phase-contrast microscopy before hybridization to ensure sufficient spreading of chromosomes. The probe complementary to LTR and Gag region of the integrating vector was labeled by nick translation using Spectrum Green-dUTP. Labeled probe was purified before hybridization to remove unincorporated nucleotides. Slides and probes were denatured before hybridization. Hybridizations were performed in a standard hybridization buffer for 18 hr. Slides were washed in a low-stringency washing buffer, mounted, and visualized by fluorescent microscopy.

Size exclusion chromatography. BioSil 125 high-performance liquid chromatography (HPLC) column (BioRad) was equilibrated 50 m*M* phosphate buffer containing 10% 10 m*M* TRIS buffer, pH 6.8, and subsequently calibrated with the following marker proteins: soybean trypsin inhibitor (20.1 kDa), bovine cationic anhydrase (29 kDa), chicken egg albumin (45 kDa), bovine galactosidase (68 kDa), human apo-transferrin (80 kDa), and blue dextran (500 kDa). The loading volume was 0.1 ml; flow rate, 5 ml/min; and pressure, <20 bars. For semipreparative use, the loading volume was increased to 0.8 ml, resulting in substantial peak widening but without deteriorating the EPO-peak recovery, which took 1.5 min, corresponding to 7.5 ml fraction.

DEAE ion exchange chromatography. It was performed using DEAE cellulose HPLC ion exchange column (Waters). The column was equilibrated 50 m*M* phosphate buffer containing 10 m*M* TRIS, pH 6.8. For elution, a linear gradient of the same buffer containing 2 *M* NaCl was used.

BioAnalyser 2000 protein 230 chip assay. It was performed according to the instructions in the user guide insert.

Amino acid composition. Our standard hydrolysis procedure employs 6 N HCl for 24 hr at 110°C. L-8800 Hitachi analyzer was used to determine amino acid composition of the EPO samples. The analyzer utilizes sodium citrate buffer system. Ion-exchange chromatography was used to separate amino acids. The separation was followed by a postcolumn ninhydrin reaction detection system quantifying individual amino acids to picomole level (~100 pm).

Matrix-assisted laser desorption/ionization–time of flight analysis

A Bruker Autoflex II matrix–assisted laser desorption/ionization–time of flight (MALDI-TOF) was used for molecular weight determination. MALDI is a soft ionization technique for the analysis biopolymers with a mass range of 500–300,000 Da. The sample was spotted after mixing it (1:2 ratio) with a sinapinic acid matrix and allowed to dry at room temperature. The instrument was operated in linear mode for better sensitivity. Data acquisition and processing was controlled by Flex Control and the spectra analyzed using Flex Analysis Version 3.0.

Peptide mapping

Tryptic digest was prepared by digesting 200 μl of EPO solution (0.50 mg/ml) in ammonium hydrogen carbonate (100 m*M*) with 2% trypsin for 8 hr at 37°C. Triflouroacetic acid was added to a final concentration of 1% at the end of incubation time. Tryptic digests were analyzed by LTQ Orbitrap mass spectrometer (Thermo Electron), interfaced to a Prominence 2D Nano HPLC system (Shimadzu Scientific Instruments) through an Advance Nano Spray Source (Michrom Bioresources). Each sample was injected onto a reverse-phase trapping cartridge (Targa C18, 5 μm, 2.5×0.5 mm; Higgins Analytical) at 20 μl/min with solvent A and separated with an Magic C18 AQ nanoLC column (5 μm, 0.2×50 mm; Michrom Biosciences). The samples were chromatographically separated using a binary solvent system consisting of A, 0.1% formic acid and 2.5% acetonitrile, and B, 0.1% formic acid and 97.5% acetonitrile at a flow rate of 1 μl/min. A gradient was run from 10% B to 45% B over 35 min. The mass spectrometer was operated in positive ion mode with the trap set to data-dependent MS/MS acquisition of the top 5 ions with charges >1. The instrument was set to complete a full mass scan from 400–2,000 Da at 600,000 resolution in the orbitrap, followed by data-dependent MS/MS analysis of up to five most intense ions in the linear ion trap at unit mass resolution. Peaks eluting from the liquid chromatography column that have ions above 5,000 arbitrary intensity units and charges higher than 1 trigger the ion trap to isolate the ion and perform an MS/MS experiment scan after the MS full scan. Data files created were searched against IPI Human data base through Mascot and Sequest search engines. Search results were integrated into a Scaffold file for evaluation.

Statistical analysis

For basic statistical calculations and for the two-sided T-test, Prism software (version 5.03, Graphpad Software, Inc.) was used.

Results and Discussion

Erythropoietin

An LV expressing the human *EPO* gene from the human elongation factor 1 alpha promoter (Laubach *et al.*, 1996) (pLTG90) was constructed (Fig. 1A). CHO DG44 cells were transduced with the cognate LV that resulted in accumulation of high number of transgene copies per cell. The bulk transduced cell culture set 1 and set 2, respectively, showed 62 and 92 vector copies per cell by PCR. The high copy number was confirmed by FISH using a probe that recognized vector-specific sequences. As many as 6 randomly scattered copies of the transgene were seen in some chromosomes (Fig. 2B and C), while control parental CHO DG44 cells were negative for fluorescence (Fig. 2A). A high-contrast reverse image of one of the cells shows clearly the multiple integration sites of the vector (Fig. 2D). The bulk culture was used for single-cell cloning by limiting dilution, and clones were identified, screened, and ranked within 4 weeks after transduction (Fig. 3).

The clones were ranked by volumetric EPO productivity (Fig. 3A). A typical clone produced 50–250 mg/liter, while the low-producer clones secreted in the 1–10 mg/liter range. The clones producing more than >1,000 mg/liter were considered high producers. The frequency of these clones reached approximately 5% in the population analyzed.

We evaluated volumetric productivity by inoculating CL-1000 tissue culture flasks with seeding 5×10^6/ml cells of four high-producer clones. The clones exhibited high viability (95–96% throughout the culture period), with cell concentrations reaching up to $40–60\times10^6$/ml. The supernatants were harvested every 3 days, and the cell concentration was reset to $20–30\times10^6$/ml if this value was exceeded in the previous culture period. The medium was monitored for glucose consumption, pH change, and NH_4^+ and lactic acid accumulation to ensure that the cell viability was maintained near 95%. The supernatants were tested for EPO by enzyme-linked immunosorbent assay (ELISA). Figure 4B shows the productivity in the selected cell harvests. E-104 had a median EPO production of 4,200 mg/liter, while the other three clones (E-108, E-110, and E-118) had a higher median productivity: 13,000 ± 2,800, 16,500 ± 6,800, and 14,000 ± 9,000 mg/liter, respectively (Fig. 3B).

The supernatant from each EPO-producing clone was harvested and tested for purity and apparent molecular weight by capillary electrophoresis (BioAnalyser 2000, Protein 230 sensor chips). The purity of the protein in the supernatant was very high. The peaks corresponding to EPO dominated the detectable protein population and the purity was consistently calculated to exceed 95% (average 98% ± 1.72%). The apparent molecular weight was determined capillary electrophoresis (BioAnalyser) to be ~37.5 kDa, with a standard deviation of 0.372 (Fig. 4B and C). The observed apparent molecular weight is consistent with previous data, which suggests that the expected apparent molecular weight for a fully glycosylated EPO (at 37.5 kDa) results from the glycosylation and the additional interaction of heavily glycosylated EPO with the gel matrix (Boissel *et al.*, 1993; Yuen *et al.*, 2003). The apparent molecular weight was confirmed by Western blot analysis (Fig. 4A), where the EPO samples migrated at a molecular weight corresponding to the purified, fully glycosylated human recombinant EPO standard (Sigma; size exclusion chromatography purified by manufacturer). The very low-standard deviation of the apparent molecular weight values over the set of EPO supernatant samples produced by four

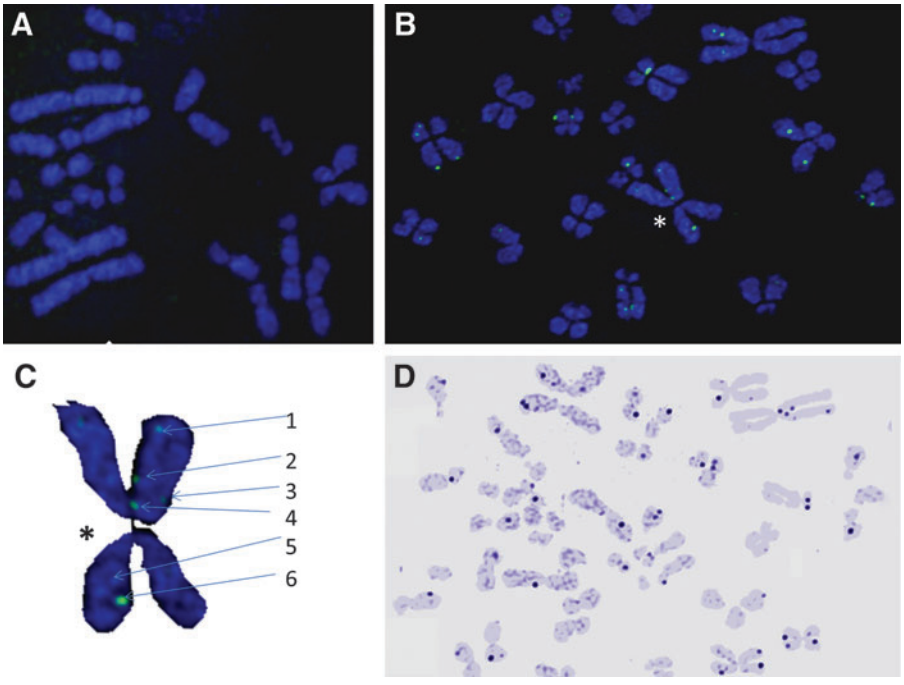

FIG. 2. Fluorescent *in situ* hybridization staining for lentiviral vector insertion sites in Clone E-118. Cells were harvested, treated with hypotonic solution, and fixed in methanol/acetic acid. Metaphase spreads were prepared for the DG44 **(A)** and DG44 clonal cell line E-118 **(B–D)**. The probe complementary to LTR and Gag region of the integrating vector was labeled by nick translation using Spectrum Green-dUTP. While the DAPI counterstained chromosomes in the parental cell line had no insertions **(A)**, a large number of insertions were seen in different spreads of erythropoietin-expressing E-118 clone **(B)**. An enhanced contrast, color-inverted view of the same clone is shown **(D)**. Many of the chromosomes had one or more vector insertions, and **(C)** the enlarged chromosome view shows at least six separate insertions within a single chromosome.

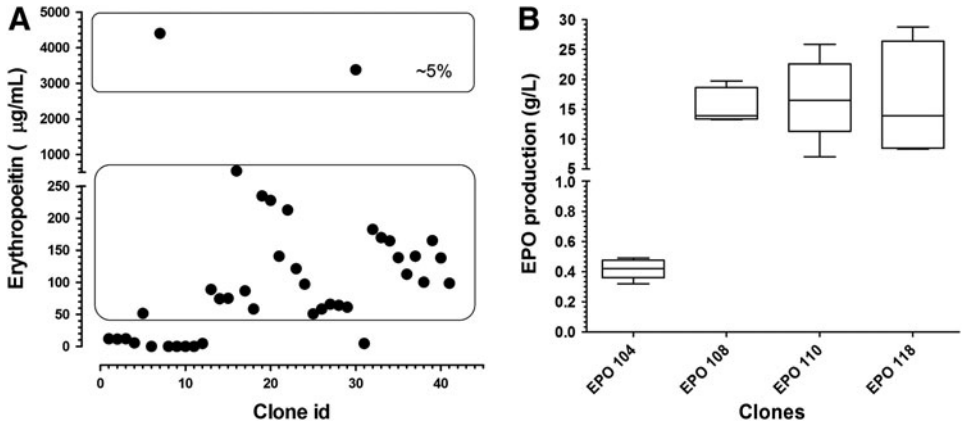

FIG. 3. Productivity of erythropoietin-secreting single-cell clones. After cell transduction, the majority of the cells produced 50–250 μg/ml of erythropoietin in 96-well plates **(A)**. The overall frequency of the high-producer cells in the single-cell cloned population reached almost 5%. **(B)** Four of the selected clones grown at high densities in perfusion culture (CL-1000 flasks, in ProCho5 medium, 37°C), which achieved consistently high levels of erythropoietin production over four different harvests.

different clones, which varied by passage number, density, and time of harvest (specific conditions not shown), strongly suggests substantial stability and reproducibility of recombinant EPO production from cell substrates genetically modified with LVs.

To verify and confirm the identity and glycosylation pattern of EPO produced from LV-modified cells, we purified the protein from the supernatant of clone E-118. The high EPO concentration and initial purity in the supernatant (>95%) allowed us to abbreviate many steps of the purification process by simply combining size exclusion chromatography with a second buffer exchange step (Fig. 5A). Western blot analysis confirmed that the majority of the EPO was eluted in the second peak at 7.1 min (Fig. 5B, SEC-P2 lane). The first peak had a small amount of aggregated EPO (data not shown). Fractions were collected after size exclusion chromatography and eluted with a linear salt gradient. After elution, EPO was primarily localized in the first and largest peak (>95% of the total eluate peak areas; Fig. 5B, IEX-P1, P2, and P3 lanes). The purity of this peak was analyzed using the Protein 230 chip on BioAnalyser apparatus. The data showed that the purity of the EPO fraction (P1) was at least 99.3% (Fig. 5C and D).

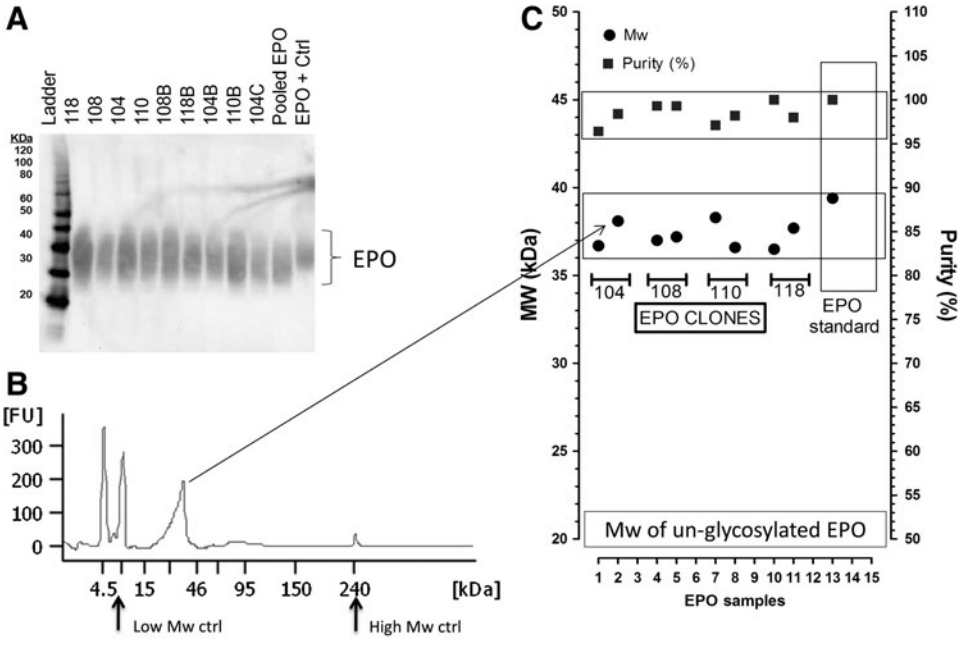

FIG. 4. Analysis of erythropoietin production in high-density cell cultures. Supernatants from randomly selected cell clones were tested for erythropoietin by Western blot analysis (4–12% Bis-TRIS Gel, MOPS, 1:1,100 Rabbit antierythropoietin IgG, and 1:10,000 diluted donkey antirabbit IgG HRPO). The reference purified erythropoietin sample (Sigma) is shown in lane 12 of **(A)**. All of the supernatants from the various erythropoietin clones displayed highly consistent bands between 30 and 40 kDa. This apparent molecular weight was confirmed by BioAnalyser capillary electrophoresis **(B** and **C)**. The apparent molecular weight was consistently and reproducibly between 36 and 38 kDa in all clones analyzed. This consistency paired with high purity of the supernatants resulted in less than 5% of the total protein content appearing outside of the erythropoietin band.

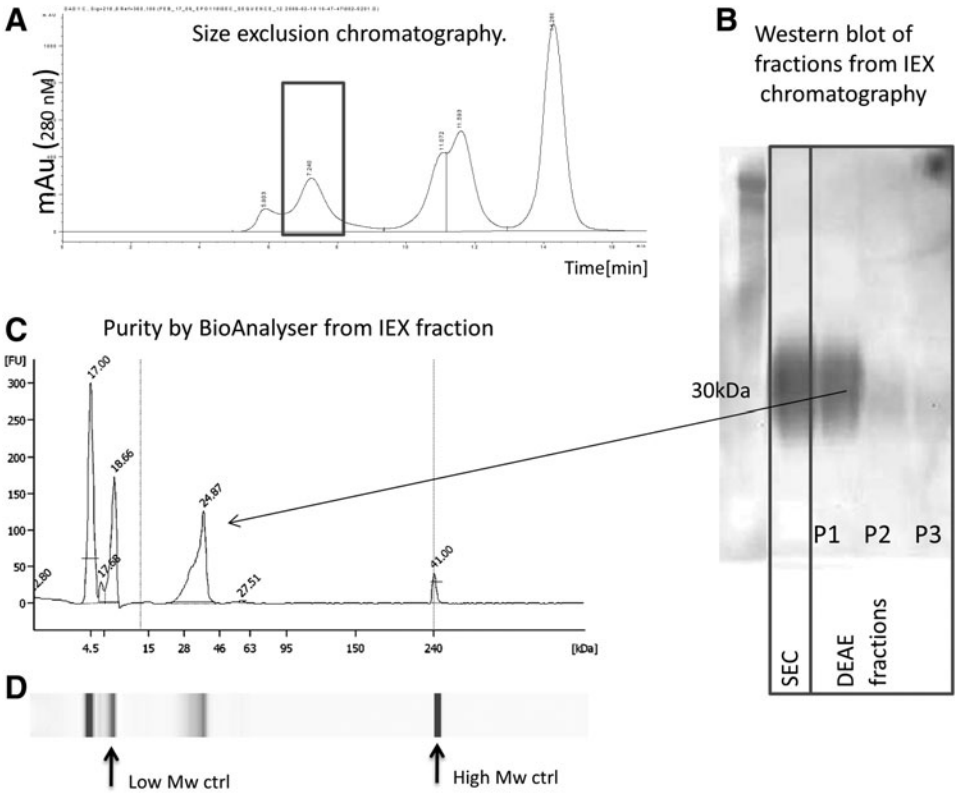

FIG. 5. Purification of recombinant erythropoietin. The purity and high concentration of erythropoietin in the supernatant enabled purification to start with simple size exclusion chromatography (0.7 ml onto a BioSil 125 HPLC column; BioRad) equilibrated with 50 m*M* phosphate buffer and containing 10% 10 m*M* TRIS **(A)**. The void volume contained a minor amounts of protein consisting mainly aggregated erythropoietin, which was indistinguishable from monomeric erythropoietin by capillary gel electrophoresis (BioAnalyser) or by Western blot (data not shown). This initial step also serves as buffer exchange with the collected fraction directly loaded into DEA Cellulose ion-exchange column, which was eluted with linear salt gradient. The resulting three peaks (P1, P2, and P3) all contained erythropoietin isomorphs, separable by ion-exchange chromatography, as shown by Western blot analysis (4–12% Bis-TRIS Gel, MOPS, 1:1,100 rabbit antierythropoietin IgG, and 1:10,000 diluted donkey antirabbit IgG HRPO) **(B,** lanes 2, 3, and 4, respectively**)**. The purity of the main fraction (P1) was determined using BioAnalyser capillary electrophoresis. The two-step purification achieved 99.3% purity **(C** and **D)**, sufficient for detailed peptide analysis and mass spectrometry of the sample.

The identity of the purified EPO was confirmed by tryptic digestion of the protein followed by mass spectrometry analysis. We were able to identify four peptides from the tryptic digest as peptides derived from EPO and confirm the sequence identity of the protein. The four peptides with their *M/z* values in parentheses were MEVGQQAVEVWQGLALL SEAVLR (81), EAISPPDAASAAPLR (144), SLTTLLR (131), and TITADTFR (159). None of the theoretical *M/z* values present in the tryptic digest corresponded to any peptides containing the N-glycosylation sites, indicating that all those sites were postsynthetically modified. Only the peptide EAISPPDAA**S**AAPL (144) that contained the amino acid 153S (the only potential O-glycosylation site in EPO) was found at the theoretical *M/z* value. This means that this peptide was either unmodified or that the more sensitive o-glycosylation was destroyed under the conditions required for tryptic digestion and the subsequent mass spectrometry (Liu *et al*., 2009; Zheng *et al*., 2009). Notwithstanding this result, it is known that the o-glycosylation at 153S is less efficient (O'Connell and Tabak, 1993) and the lack of glycosylation does not hinder the secretion or affect the biological effects of EPO (Delorme *et al*., 1992). The MALDI-TOF spectrum of purified EPO was also obtained and the molecular weight determined to be 25,900 Da. This is 7,869 Da more than the calculated theoretical molecular weight of 18,031 Da for the secreted EPO, which lacks the leader peptide and is deficient in the carboxyterminal three amino acids of the native sequence. This indicates that extensive post-transcriptional modification (N-glycosylation) is taking place, achieving an apparent 43% glycan/protein ratio, although we have no data on the microheterogeneity of the glycosylation. Considering that the published ratios for EPO are close to 40%, the EPO in our harvests can be considered fully glycosylated; the three N-Acetyl glycosylation sites fully occupied and the glycans have the acceptable average molecular weight range.

The stability of the cell lines was assessed by comparing clones from the second passage and after maintaining the corresponding cell line for over 5½ months with nearly biweekly serial passages at a ratio of 1:10 in completed ProCho5 serum-free medium, without any selection or re-cloning. One million cells from each of the four selected clones were plated in triplicate using cells from the early passage and from cells cultured for 5½ months, in 12-well

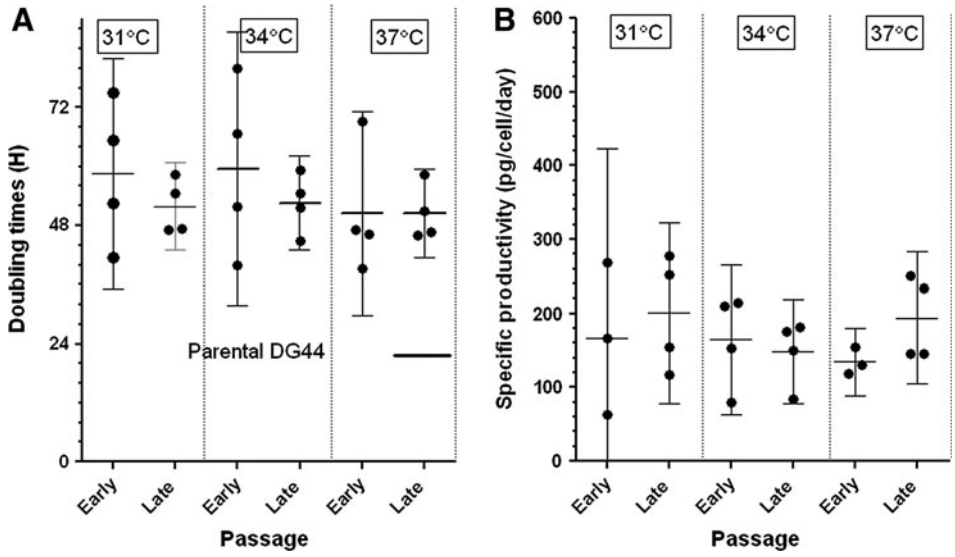

FIG. 6. Stability of erythropoietin-producing cell lines. The selected erythropoietin-producing clones (104, 108, 110, and 118) were maintained by serial passaging in serum-free ProCho 5 medium for over 5½ months (22 passages). It was determined whether the growth characteristic or specific productivity of the cells changed significantly during this period. **(A)** The cell lines showed no or minimal temperature dependence, the range of the observed doubling times remained similar, and there was no trend toward shortening or lengthening of the doubling times at any temperature. The only trend that can be seen is a more consistent growth, stabilizing around 50–60 hr. This is more than two times longer than the doubling time of the parental DG44 cell line. **(B)** The specific productivity (directly measured from supernatant using BioAnalyser) varies from cell line to cell line, but does not decline over time. The cells from late passages preserved their ability to secrete 100–200 pg erythropoietin daily.

tissue culture plates and kept at 37°C, 34°C, or 31°C for 4 days (36 cultures in total). The cells were counted at the end of the fourth day and samples were taken from supernatants to measure the EPO content, apparent molecular weight, and purity using the BioAnalyser apparatus. The overall viability of the cells after 4 days of culture was >93% (96.3% ± 2.79%). The average purity of EPO (expressed as a percentage of total protein in the supernatant) was 98.1% ± 0.798% and did not change with the number of passages. To look for possible trends, we calculated the mean doubling times of the cells for the four cell lines. With increasing passage over the 5½ months of culture, there was a tendency to converge the doubling time variance from 56.2 ± 14.3 to 51.6 ± 5.3 hr (Fig. 6A), but this change was found not to be significant in a paired *t*-test. Notwithstanding the number of passages, the doubling times were much longer than the parental DG44 cell line (23 hr). The doubling time data showed concordance with the mean specific productivity of the cells. Cells from early passages demonstrated an average EPO production at a remarkable 156 ± 63 pg/cell/day, which was maintained at 180 ± 60 pg/cell per day after 5½ months of culture. These results demonstrate *in toto* that neither the high vector copy number nor high levels of EPO expression were toxic to the cells (Fig. 6B). Cell growth or EPO productivity was compared at varying temperatures (31°C, 34°C, 37°C; Figs. 6A and 7B), but this range of temperatures did not appear to significantly affect the protein productivity of the cultures. The lack of temperature dependence could be because of the cells having reached their upper limit of production at 180 ± 60 pg/cell per day.

To demonstrate the safety of using LVs for the genetic modification of cell substrates, we investigated the level of LV *gag* protein by p24 ELISA. Since the vector is defective and does not encode any LV proteins, and the cell line has gone through numerous divisions from initial transduction, there should be no LV proteins present in the supernatant of the cell lines. The p24 ELISA detects the LV gag protein and is a highly sensitive assay for detection of LV particles (see Materials and Methods). The p24 protein was not detected in any of the raw supernatants from the four different cell culture clones that were maintained for 5½ months, or in the purified EPO samples (the limit of detection level for the p24 ELISA assay was 3.4 pg/ml). Finally, we determined whether the level of remnants of the integrated LV DNA in the final product was higher or lower to that typically seen for residual cellular DNA. We found that the levels of LV DNA (as assayed using quantitative real-time PCR for HIV *gag* sequences; data not shown) were below those typically seen with cellular DNA (< 10 ng of DNA per dose, assuming a daily 5,500 units (or 32 μg) of EPO per dose (Delorme *et al.*, 1992; O'Connell and Tabak, 1993; Schroder *et al.*, 2002).

Recombinant human–porcine factor VIII

Factor VIII is normally a difficult protein to express (Lusher *et al.*, 1993). We developed an LV (PLTG1133) that expressed a chimeric human–porcine factor VIII gene that was driven by the human elongation factor 1 alpha promoter. The rhp FVIII protein has improved characteristics over native human factor VIII (Doering *et al.*, 2002). To compare the transcriptional efficiency of LV-mediated gene delivery with conventional plasmid DNA gene delivery, an LV construct was either transfected with plasmid DNA or transduced with an LV

expressing the *rhpFVIII* gene (Doering *et al.*, 2002) (ET-801; Fig. 1B). The transcript mRNA was identified with Southern blot analysis, and the transcripts were found intact and of expected size. The plasmid-transfected BHK cells were selected in antibiotic, while the lentivector-transduced (MOI = 150) BHK clones were transduced under conditions that assured comparable number of inserted copies of recombinant genes per cell. Higher levels of rhpFVIII production can be attained with more optimized transduction conditions using LVs (data not shown). Using limiting dilution, the top 24 rhpFVIII-expressing clones were selected and analyzed further for fVIII expression, rhpFVIII RNA levels, and proviral copy numbers of rhpFVIII-encoding vectors. As shown in Fig. 7A, rhpFVIII protein activity in the LV-transduced cells' supernatant (21 clotting units/10^6 cells/24 hr, median) was significantly greater than that of the plasmid-transfected cell lines obtained after selection (6.6 units/10^6 cells/24 hr; $p < 0.0001$). This represents at least a threefold increase in expression over plasmid-transfected cell clones (Fig. 7B; a mean of 2.7 versus 3.2 copies/diploid genome; $p = 0.2411$, a not significant difference). Analysis of steady-state fVIII mRNA levels (Fig. 7C) indicated a concomitant threefold increase in transcript numbers per cell in the clones obtained by using LVs over plasmid-transfected cell clones (7,600 ± 1,500 vs. 1,600 ± 250; $p < 0.0001$). The data demonstrate that the improved mRNA and specific protein productivity are the consequence of improved transcription from LV-transduced clones (nearly 5:1; Fig. 7D; $p < 0.0001$) and not the selection of BHK cell lines with different fVIII production capabilities since the activity per transcript ratios are almost identical (Fig. 7B). This indicates that improved fVIII production is the result of the predilection of LVs to insert themselves into transcriptionally active regions of chromatin (Schroder *et al.*, 2002) and not some intrinsic property of the selected cell clones. Therefore, one important advantage of LV-modified cell substrates over conventional plasmid DNA transfection is the transcriptional

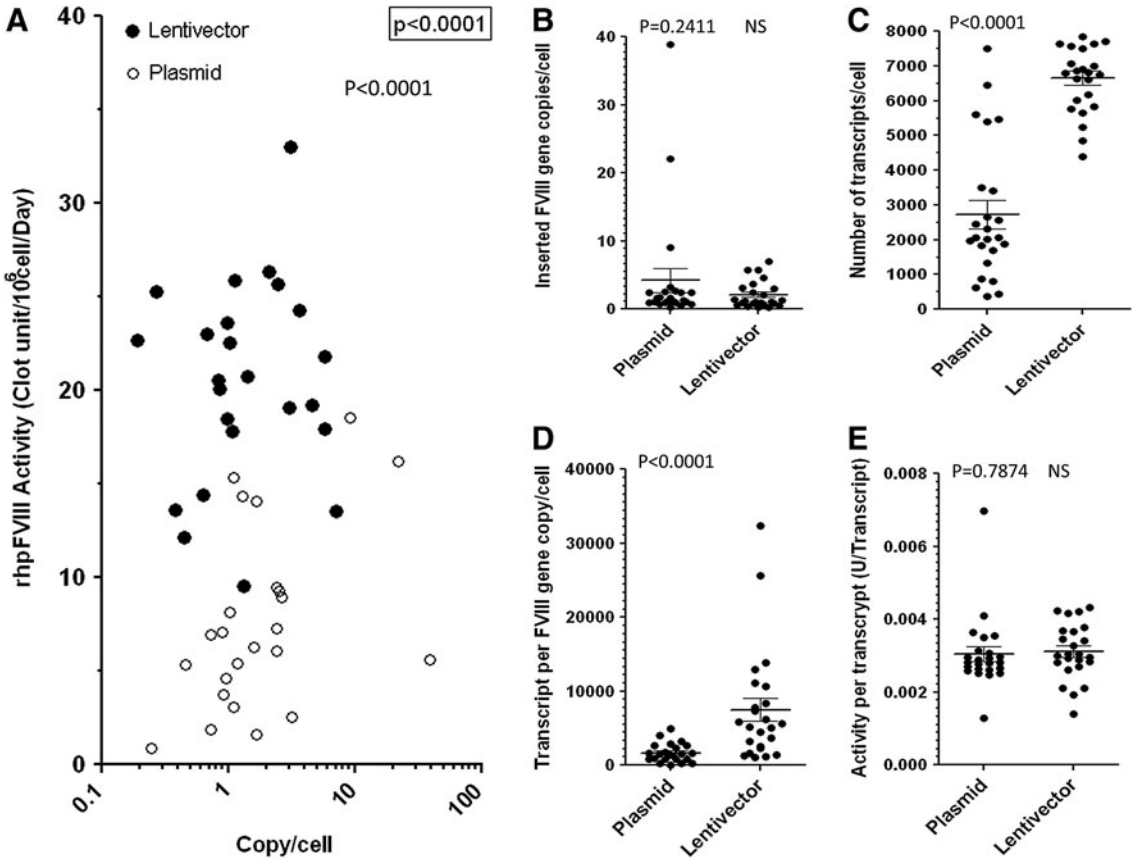

FIG. 7. Transfection or transduction of BHK cells with rhpFVIII encoding plasmid or lentivector. A total of 24 high-expressing clones were selected from each group for analysis of fVIII transgene copy number and expression of protein. As shown in **(A)**, the median rhpFVIII activity measured in the supernatant from cell lines generated using lentiviral vector was 21 clot units/106 cells/24 hr, while the median plasmid-based fVIII expression was 6.6 units/10^6 cells/24 hr ($p < 0.0001$). **(B)** Similarity of transgene copy number between plasmid and rhpFVIII cell clones (2.7 vs. 3.2 copies of rhpFVIII gene/diploid genome; $p = 0.2411$, a nonsignificant difference). **(C)** Steady-state rhpFVIII mRNA levels in the clones, demonstrating a threefold median increase in the number of mRNA transcripts in the clones generated by using lentiviral vectors (an average of 6,500 transcripts/cell) as compared with plasmid-derived fVIII expression of 2,560 transcripts/cell ($p < 0.001$). The transcript/inserted copy ratio for the lentiviral vector-transduced cells is significantly higher in the lentiviral vector-derived clones than in the plasmid-derived clones (7,600 ± 1,500 vs. 1,600 ± 250; $p < 0.0001$). As expected, the cells performed similarly in the synthesis and secretion of rhpFVIII, as there were no detectable difference in the produced FVIII activity per unit mRNA regardless of the type of vector used **(E)**.

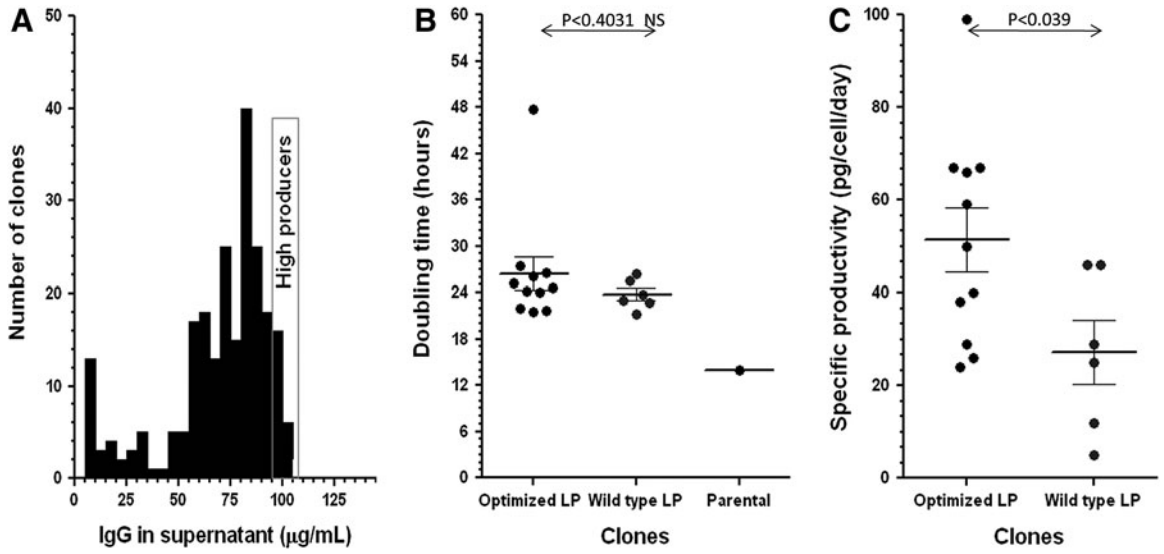

FIG. 8. Cloning antihuman CD20 antibody-producing cell. Chinese Hamster Ovary cells were transduced with a 1:2 mixture of 100 multiplicity of infection of human CD20-specific IgG heavy- and light-chain encoding plasmids, using 5×10^3 cells in 0.1 ml of ProCho5 medium repeatedly. The heavy-chain gene was used in two variants: one had wild-type leader peptide (W) and the second had a proprietary leader peptide optimized to match the recombinant humanized heavy chain (L). Single cells were cloned from the respective bulk cultures by limiting dilution into 96-well plates, and volumetric IgG productivity **(A)**, doubling times **(B)**, and specific IgG productivity **(C)** were determined from individual cell cultures in log growth phase, run in triplicates. Out of over 600 clones, only less than 220 were tested to obtain the volumetric productivity dataset since the distribution curve fell very sharply at the high-producer end, and he probability to find a significantly better clone declined precipitously. High producers of both versions of IgG were selected.

efficiency of each integrated transgene because of integration site selection.

Anti-CD20 antibody

Three LVs expressing the humanized anti-CD20 antibody kappa light chain and gamma heavy genes driven from the SCMV promoter were constructed (Fig. 1C). CHO cells were transduced with a 1:2 ratio of LV anti-CD20 IgG heavy-chain-expressing and LV anti-CD20 IgG light-chain-expressing vectors. In an independent study, we found that a light-/heavy-chain ratio at, or above, 2 seems to be optimal for effective IgG production, as other ratios often resulted in intracellular accumulation of intermediates creating bottlenecks in the assembly of heavy- and light-chain complexes (data not presented). CHO cells were transduced with two LVs, each encoding the human anti-CD20 IgG heavy and light chains. Two variant heavy-chain genes were used: one had wild-type leader peptide (W) and the second had an optimized leader peptide (O, Fig. 1C). Single cells were cloned from the respective bulk cultures by limiting dilution into 96-well plates, and volumetric IgG productivity was determined for 220 clones (Fig. 8A). High expressors were selected, and the mean specific productivity was determined to exceed 60 pg/cell/day (the highest being ~98 pg/cell/day), a remarkably high level when compared with other reported averages (Bebbington *et al*., 1992; Kanda *et al*., 2006; Beyer *et al*., 2009). The steep decline of the productivity distribution curve indicates that the productivity limits may have been reached and pushing transcriptional productivity higher may not increase immunoglobulin levels in the medium. Other bottlenecks, such as the efficiency of IgG secretion, may exist. This is supported by the observation that the average doubling time of the parental cell line (about 13 hr) has nearly doubled in the isolated clones (Fig. 8B). We examined this issue by comparing a wild-type leader peptide and an optimized leader peptide to determine if the more optimized leader peptide could increase antibody productivity by relieving bottlenecks for protein secretion. We found that the optimized leader sequence nearly doubles the specific productivity of the cells (Fig. 8C). This shows that while LVs can increase protein productivity by increasing the number of transgene transcripts produced within cells, other protein production bottlenecks can exist, which can be relieved with appropriate vector and cell line engineering.

Conclusions

Developing genetically modified mammalian cell lines for protein production has typically required considerable investment of time and resources. Plasmid DNA transfection has been the most widely used method. This method is very inefficient and results in only a small number of cells exhibiting an integrated genome. As a result, plasmid vectors need to contain a drug resistance gene for selection of genetically modified cells. Furthermore, since plasmid DNA transfection does not produce cells with high integrated copy numbers immediately after transfection, gene amplification strategies are frequently required. All of these methods are time and resource consuming. Even so, the published production results for levels of protein production are typically in the range of 50–250 mg/liter protein in 4–7 days of long-

stirred batch cultures or 500–1,000 mg/liter in 7–10-day cultures, comparable to the amounts produced by other, nonmammalian systems (Schmidt, 2004; Grillberger *et al*., 2009). Furthermore, these methods are prone to several important shortcomings, including arduous screening of thousands of clones to identify high producers, gene silencing of high producer clones because of methylation at specific sites on the chromosome (Wurm *et al*., 1992), deglycosylation, and other post-transcriptional variations in high-producer clones (Robinson *et al*., 1994; Flesher *et al*., 1995; Lifely *et al*., 1995).

In this article, we show consistently high levels of production of EPO, a clinically relevant, but difficult-to-express factor VIII and an antihuman CD20 antibody from cell substrates after their genetic modification with an LV. Selected EPO clones were able to produce the protein up to 100–200 pg/cell/day, which translated to 10–20 g/liter when the cells were grown under high-density conditions. We were also able to generate cell clones that efficiently produce rhpFVIII, with clones reaching 100 clotting units/ml of culture, representing 21–40 clotting units per 10^6 cells/day. Furthermore, we were able to isolate cell clones producing an anti-CD20 antibody (Rituximab), with specific productivity rates up to 100 pg/cell/day.

We found that this high level of production was caused by higher transcriptional levels of transgene mRNA in cells that were transduced with LV, rather than with conventional plasmid transfection methods. This is consistent with the known integration patterns of LVs, which are known to insert into areas of high transcriptional activity (Flesher *et al*., 1995; Schroder *et al*., 2002). Therefore, cell substrates generated with LVs result in transgenes with higher transcriptionally activity than with traditional methods, such as plasmid DNA transfection. This increased transcriptional activity resulted in the higher levels of protein production.

The high levels of protein production were achieved without compromising the time taken to generate highly productive cell clones, cell substrate stability for protein production, or the uniformity of post-transcriptional modification. Highly productive cell lines could be comfortably generated within 2 months. Because of high production levels, the purity of the protein in the cell harvest was found to be very high (93–95% for EPO). This permitted development of an abbreviated purification method. Abbreviating downstream processing methods could have important implications for large-scale protein manufacturing particularly when considering improvements being attained with high-capacity chromatographic methods. Highly productive (100–200 pg/cell/day) clones were shown to be remarkably stable for 5½ months of culture with no observable gene silencing. This is in contrast to plasmid-transfected cell lines, which are known to be prone to gene silencing. We believe that the high stability afforded by LV-generated cell lines is because of the distributive nature of LV integration of transgenes. Cells transfected with plasmid DNA concatomerize or amplify the transgenes on one site of the chromosome, while LV-transduced cells distribute their transgenes throughout the cell's open chromatin. Therefore, since any one site of the cell's genome is prone to gene silencing, plasmid-transfected cell clones are highly prone to gene silencing and loss of a productive clone, since all the transgene copies are located at one site. In contrast, LV-transduced cells distribute their copies throughout the genome, limiting the effects of gene silencing to a small proportion of transgenes, while maintaining the bulk of protein productivity. All high-producing EPO clones examined displayed similar high levels of glycosylation independent of passage number, temperature, or cell density in the culture. Similar results were seen with cell clones expressing human–porcine factor VIII. These data indicate that LVs are capable of generating cell substrates that produce highly uniform post-transcriptionally modified proteins that retain high activity.

Clinical utility of LV-modified cell substrates will require purification methods that remove LV DNA from the final biologic product. This goal is made easier because LV-engineered cell substrates result in higher levels of protein production without vector-mediated cell toxicity, resulting in lower contaminating cellular protein and DNA levels in the bulk harvests and thereby potentially simplifying purification of the final product.

In toto, we describe the use of LVs for the efficient generation of cell lines for stable, high, and uniform production of three clinically relevant proteins. As therapeutic proteins transition from unique, IP-protected medicines to biosimilars, the economics of their production will become increasingly relevant. Technologies that reduce cost and increase productivity will gain importance. We propose that the genetic modification of cell substrates with LVs could be valuable in this respect.

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Author Disclosure Statement

The authors declare that they have no competing interest in respect to publication of the above article. Lentigen Corporation and Expression Therapeutics are for-profit companies that are developing the technology contained in this publication.

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Address correspondence to:
Dr. Lajos Baranyi
Lentigen Corporation
910 Clopper Road, Suite 200S
Gaithersburg, MD 20878

E-mail: lajos.baranyi@lentigen.com

Dr. Boro Dropulic
Lentigen Corporation
910 Clopper Road, Suite 200S
Gaithersburg, MD 20878

E-mail: boro.dropulic@lentigen.com

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